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Genomic Imprinting and Wilms' Tumor

Thomas Moulton, MD, Wai-Yee Chung, BA, Luwa Yuan, MA, Terrence Hensle, MD, Pamela Waber, MA, Perry Nisen, MD, PhD, and Benjamin Tycko, MD, PhD

The selective loss of maternal and reduplication of paternal chromosome 11p15.5 alleles in Wilms' tumors (WTs) points to the existence of a paternally imprinted tumor suppressor gene(s) and/or a maternally imprinted dose-dependent growth-promoting gene(s) in this chromosomal region. Two reciprocally imprinted chromosome 11p15.5 genes, *H19*, a candidate tumor suppressor gene, and *IGF2*, a candidate dominant oncogene, have been well-characterized in terms of their imprinting and expression status in WTs. Here we review and extend data indicating that a majority of WTs show a bipaternal epigenotype at these loci, with *H19* inactive and *IGF2* biallelically active. This can arise either through loss of heterozygosity (LOH) or by a non-LOH pathway involving localized biallelic hypermethylation of *H19* DNA. Conversion to this bipaternal endpoint has recently been found to affect not only these two genes, but also at least one other imprinted 11p15.5 gene, *KIP2*. Since 11p15.5 LOH and biallelic *H19* hypermethylation can occur both early and late in tumor progression and since early loss is not associated with bilaterality or multifocality of WTs, these types of lesions appear to be permissive rather than rate-limiting in Wilms' tumorigenesis.

Key words: Wilms' tumor, genomic imprinting, *H19* gene

INTRODUCTION

Genomic imprinting is an incompletely understood prezygotic process which leads to reversible parent-of-origin-specific DNA and chromatin modifications which influence subsequent gene expression in the offspring. Imprinted genes are preferentially expressed from one parental allele, with genes silenced on the paternal allele usually refered to as "paternally imprinted" and those silenced on the maternal allele as "maternally imprinted." A considerable body of circumstantial evidence implicates site-specific methylation of DNA at CpG dinucleotides as a mechanism which maintains the silent status of imprinted alleles in somatic tissues [1]. Since the imprinting of at least some genes is conserved between mice and humans, it is possible that the phenomenon has some beneficial effects for the organism, but there is also good evidence that the existence of imprinting has adverse implications for tumor progression in humans. In particular, several types of malignant pediatric neoplasms show highly selective losses of specific chromosomal alleles inherited from one type of parent. This parental bias in loss of heterozygosity (LOH) has been observed most recently for chromosome 1p35-36 markers in neuroblastomas which lack *N-MYC* amplification [2], in which the lost alleles are nearly all of maternal origin, but it was originally noted for chromosome 11p15.5 markers in embryonal rhabdomyosarcomas [3] and Wilms' tumors (WTs) [4–10], in which the lost alleles are exclusively of maternal origin.

What does the selective loss of maternal 11p15.5 alleles imply for the mechanism of Wilms' tumorigenesis? One simple model proposes the existence of a paternally imprinted (maternally expressed) 11p15.5 Wilms' tumor suppressor gene. An objection which can be raised to this model is that the existence of an imprinted tumor suppressor gene which is normally monoallelically expressed and which therefore can be functionally deleted by one-hit kinetics would imply that WTs would be exceedingly frequent. However, this objection does not apply if elimination of the 11p15.5 tumor suppressor is not rate-limiting in a multistep pathway of tumorigenesis. Alternatively, as originally suggested by Wilkins [11], since LOH in WTs usually reflects loss of maternal alleles and reduplication of the corresponding paternal alleles (rather than reduction to hemizygosity), it is possible that the functionally important gene is a maternally imprinted (paternally expressed) gene with dose-dependent growth-

From the Department of Pediatrics, Division of Pediatric Hematology-Oncology (T.M.), Department of Pathology, Division of Oncology (W.-Y.C., L.Y., B.T.), and Department of Surgery, Division of Pediatric Urology (T.H.), Columbia University College of Physicians and Surgeons, New York, New York, and the Department of Pediatrics, Division of Hematology-Oncology, University of Texas Health Sciences Center, Dallas, Texas (P.W., P.N.).

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Address reprint requests to Dr. Benjamin Tycko, Department of Pathology, Columbia University College of Physicians and Surgeons, 630 W168 Street, New York, NY 10032.

promoting activity, i.e., a maternally imprinted dominant oncogene. These two models are not mutually exclusive and in fact there is evidence that both may be correct.

Recent findings suggesting the existence of "imprinting domains" in mouse and human chromosomes [12–14], in which multiple physically linked genes are subject to coordinate parental imprinting, raise the further possibility of a variant on these simple models in which multiple imprinted 11p15.5 growth-regulatory genes are subject to coordinate alterations in expression in WTs. The available data are still insufficient to resolve these alternatives or to definitively assign the identities of the growth-regulatory 11p15.5 genes relevant to Wilms' tumorigenesis, but several candidate genes, *IGF2, H19,* and, most recently, *KIP2,* have already been well-characterized. In this review we discuss and supplement data concerning the functional perturbation of chromosome 11p15.5 genomic imprinting in WTs.

PATIENTS AND METHODS

Tissue samples from WTs, together with matched non-neoplastic tissue consisting of peripheral blood, non-neoplastic kidney parenchyma or both were obtained at surgery. Southern and Northern blotting and allelotyping for chromosome 11p15.5 markers were carried out as described [10]. The marker D11S988 was amplified by polymerase chain reaction (PCR) using commercially available primers (Research Genetics, Huntsville, AL). The H22 marker is a previously unpublished CA-repeat in the H22 cosmid [10] which was amplified by PCR using the primer pair H22CA-1 GCTGTGCCACTGCACTTC and H22CA-2 GCTCCCCATCCCATCCTG; PCR cycling conditions were an initial denaturation at 94°C for 4 min, subsequent denaturations at 94°C for 1 min, annealing at 50°C for 1 min in the first 20 cycles and 20 sec in the remaining 10 cycles and extension at 72°C for 30 sec with a final extension for 5 min. The *H19* exon 1 and exon 3–5 probes were previously described [9].

RESULTS

The *IGF2* gene, which encodes a fetal peptide growth factor, is normally expressed only from the paternal allele [15–17] and the *H19* gene, which gives rise to an abundant spliced and polyadenylated but apparently untranslated fetal RNA, is normally expressed only from the maternal allele [8,9,18,19]. Both genes map within the same roughly 200-Kb interval of chromosome 11p5.5. The imprinting of *IGF2* is therefore in the correct "direction" to possibly account for a dose-dependent growth-promoting activity in WTs with paternal chromosome 11p15.5 duplications and the imprinting of *H19* is in the "direction" appropriate for a candidate WT growth suppressor gene. *IGF2* has long been considered a candidate dominant oncogene in WT based on consideration of its normal role as a fetal mitogen and the high expression of its mRNA in WTs [20,21] and *H19* RNA has tumor growth-suppressing activity in two embryonal tumor cell lines in a transfection system [22]. Both *H19* RNA and *IGF2* mRNA are highly expressed in the developing fetal kidney, with highest expression in the renal blastema, the presumptive precursor tissue of WTs [23].

Several laboratories have recently examined the expression and functional imprinting status of these two genes in series of primary WTs [8,10,24–26]. The results in these independent studies have been quite consistent: a majority of WTs show abrogation of *H19* RNA expression, associated with biallelic hypermethylation of CpG dinucleotides within the body and promoter of this gene. This inactivation of *H19* is correlated with biallelic *IGF2* mRNA expression and there appears to be a general though somewhat variable trend for an inverse relationship in the absolute amounts of RNA from these two genes [10,25], although this was less evident in one of the studies [26]. In the roughly 40% of WTs with 11p15.5 LOH, this is the expected outcome based on a stable paternal *H19* imprint—the active maternal *H19* allele has been lost from the tumor cells and the inactive and hypermethylated paternal *H19* allele has been duplicated, along with the closely linked active paternal *IGF2* allele. More informatively, a second large subset of WTs showed biallelic *H19* DNA hypermethylation and biallelic *IGF2* expression without LOH of chromosome 11p15.5 markers. From a mechanistic point of view, the observations in this second group of tumors fit nicely with a model for the reciprocal imprinting of *IGF2* and *H19* in normal tissues based on competition of the promoters of these two genes for a single downstream enhancer element [27]. According to the predictions of this model, the functional inactivation of *H19* in the tumor cells allows the previously inactive maternal *IGF2* promoter to occupy the single downstream enhancer element and thereby become active.

The hypermethylation of *H19* in this subset of WTs cannot be trivially explained as an epiphenomenon of rapid tumor cell growth and does not reflect a global derangement in DNA methylation in the tumor cells. The first point is made by our observation in two WT cases that not only the tumor samples, but also the non-neoplastic kidney parenchyma adjacent to the tumors showed biallelic hypermethylation of *H19* DNA [10]. This is illustrated more extensively Figure 1, in which two independent samples of non-neoplastic kidney parenchyma from one of the patients both show this abnormality, indicating that the epigenetic lesion was quite widespread in this kidney and suggesting that it may have occurred in one or more kidney precursor cells early in nephrogenesis. Of potential importance, in neither of these two patients' kidneys was there evidence of extensive nephrogenic rests

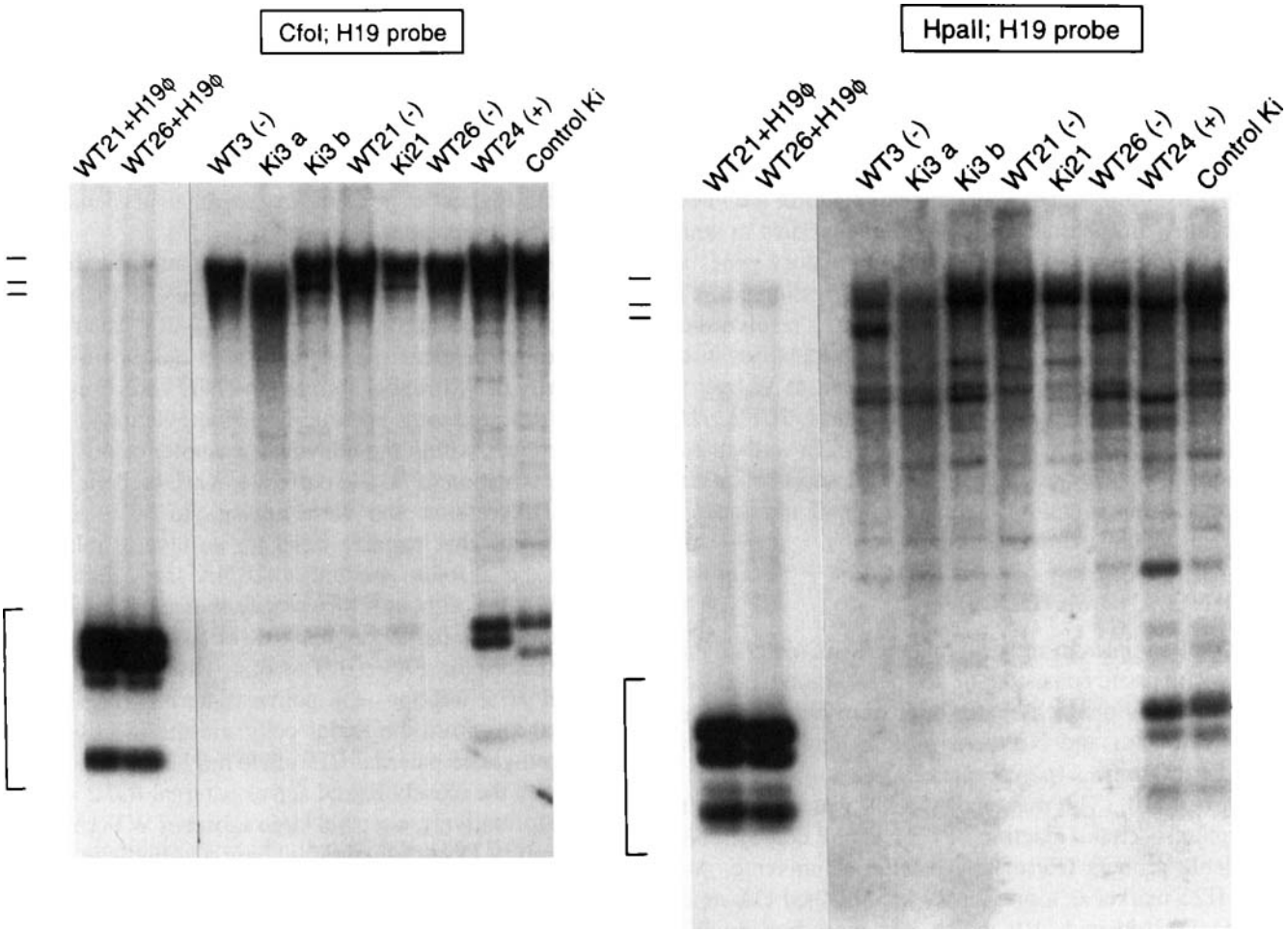


Fig. 1. Southern blot analysis of *H19* CpG-methylation in WTs and non-neoplastic kidney. Genomic DNAs were digested with the indicated methylation-sensitive restriction enzymes. The blots were hybridized with the *H19* exon 3–5 probe. *H19* hypermethylation is seen as the reduction in intensity of the bands corresponding to the small limit-digest fragments (brackets). In samples spiked with a (nonmethylated) *H19* phage clone (+H19ϕ), there is complete digestion of the cloned DNA to the limit-digest fragments. Ki3a and Ki3b are two separate tissue fragments taken from areas more than 1 cm apart in the non-neoplastic parenchyma of the kidney containing WT3. Ki21 is a sample of non-neoplastic parenchyma from the kidney containing WT21. The presence or absence of *H19* RNA in the tumors is indicated as (+) or (−). In Table I, WT3 = case 428, WT21 = case 511, WT24 = case 537. Similar results were obtained with the *H19* exon 1 probe (not shown).

or multifocal WTs and neither patient had obvious signs of Beckwith-Weidemann syndrome (BWS). The second point is made by our mapping of the region of CpG hypermethylation both in the tumor DNA and in the affected non-neoplastic kidney DNA of these two patients. The hypermethylation was only detected with probes within the body and promoter of the *H19* gene and was not present at several locations in upstream and downstream flanking DNA [10]. In addition, we have recently characterized a novel ubiquitously expressed gene, *L23MRP*, situated very close to *H19* (within 40 Kb downstream), and we have found that this gene is neither hypermethylated nor transcriptionally repressed in WTs [28].

Further, the abrogation of *H19* expression in most WTs does not simply reflect tumor cell differentiation. This point is made by the fact that, while *H19* is normally expressed highly in renal blastema, WTs of both standard triphasic and blastemal predominant histology can show inactivation of this gene [10]. While histologic features were not discussed in the other published series, since a majority of WTs in each of these series showed *H19* inactivation and since most WTs have a significant component of neoplastic blastema, *H19* inactivation is clearly not correlated with cell differentiation. Moreover, while *H19* RNA expression declines somewhat in the normal mature kidney, this is not accompanied by biallelic DNA hypermethylation. In sharp distinction with WTs, adult kidneys maintain a hypermethylated paternal allele and a persistently expressed hypomethylated maternal allele [9].

To ascertain the frequency of epigenetic inactivation

of *H19* and to ask whether this phenomenon correlates with particular clinical presentations or patterns of subregional chromosome 11p15.5 allele losses, we have doubled our original series of cases and assessed 11p15.5 allelic markers, *H19* DNA methylation and *H19* RNA expression in a group of 52 WTs (Table I). Several relevant findings emerge from this analysis. Biallelic *H19* DNA hypermethylation, defined as resistance to digestion with CfoI and HpaII restriction enzymes on Southern blots probed with both the exon 1 and exon 3–5 *H19* probes [10], was present in 77% (40/52) of the cases. Of these cases, 19 (37% of all cases) showed LOH for 11p15.5 markers, 19 (36% of all cases) retained heterozygosity for these markers and 2 were not informative. The remaining 23% (12/52) of cases showed a normal pattern of monoallelic (paternal) *H19* hypermethylation. Of these, 10 tumors retained 11p15.5 heterozygosity and two showed partial 11p15.5 LOH. RNA was available from most of the cases and of these 73% (30/41) showed no *H19* RNA expression or expression reduced more than 20-fold from fetal kidney levels. Three tumors expressed *H19* RNA at about 10-fold less than fetal kidney and 8 tumors (20% of cases) showed high *H19* RNA expression, comparable or exceeding fetal kidney levels. A representative Northern blot of *H19* RNA in a random series of WTs, all of which are cases which have been obtained since our previous two studies [10,28], is shown in Figure 2. This shows both the high frequency of *H19* inactivation in WTs and, by reference to the data in Table I, the good correlation of this phenomenon with biallelic CpG hypermethylation of *H19* DNA. Only two cases (535 and pw395) did not follow this pattern; both were WTs which, despite the lack of CpG hypermethylation, nevertheless showed very low *H19* RNA expression.

With respect to the *IGF2/H19* locus, the functional endpoint of chromosome 11p15.5 LOH is the same as that of the *H19* hypermethylation pathway, i.e., conversion to a bipaternal epigenotype. But this does not prove that either of these two genes is the most important target of 11p15.5 LOH in Wilms' tumorigenesis. To attempt to map a minimal region of 11p15.5 allelic loss in our series of WTs we have typed polymorphic markers spanning a roughly 14 cM region centered around *IGF2/H19* (Table I). Most of the cases were informative at the distant flanking markers D11S988 (CA-repeat; 39 informative cases) and *HRAS* (combined TaqI-VNTR and CGG-repeat markers; 25 informative cases) and no example of subregional LOH was found; all informative cases with LOH for markers in or near *IGF2/H19* also showed LOH for the flanking markers and all informative cases with retention of heterozygosity for *IGF2/H19* also retained heterozygosity for the distant flanking markers. A similar lack of subregional 11p15.5 LOH has been observed in other substantial series of WTs [29]. It may be that analysis of additional cases will pinpoint a smaller minimal region of recurrent LOH which must contain the most important target gene(s) for tumorigenesis, or alternatively it may be that more than one 11p15.5 gene must be affected to generate the WT phenotype. In fact, a third imprinted gene with predicted growth-regulatory activity, that encoding the cyclin-cdk inhibitor $p57^{KIP2}$(30), is also dysregulated in a subset of WTs (31,32), and we have found that WTs with *H19* inactivation also frequently inactivate *KIP2,* while those with persistent *H19* expression also show persistent KIP2mRNA (32).

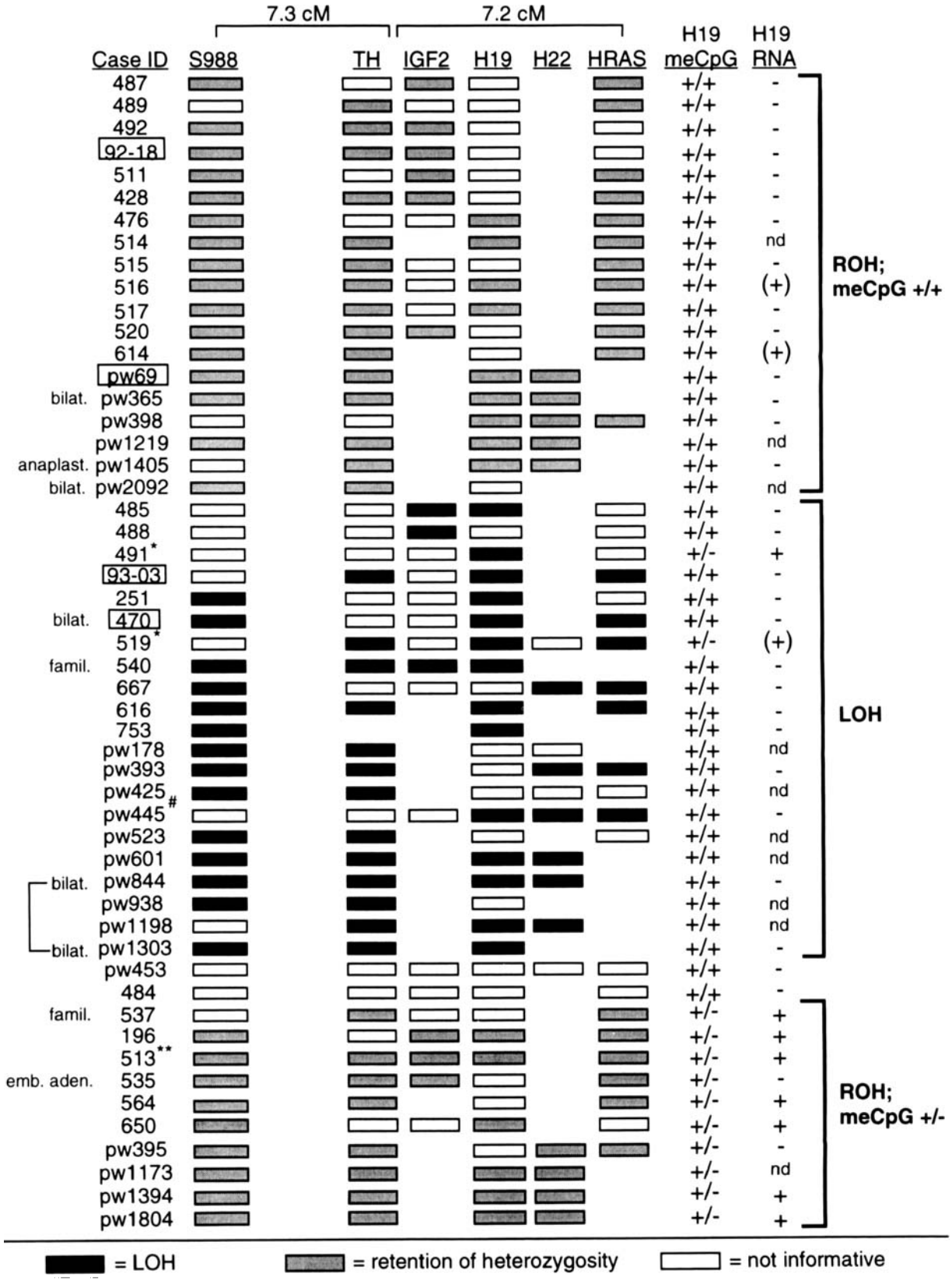
Since an imprinted tumor suppressor gene can in theory be eliminated via a "one-hit" pathway, one objection to the existence of such genes is that the predicted frequency of tumors caused by their loss would be much higher than the observed frequency. This objection can be answered if functional elimination of the imprinted tumor suppressor gene is not rate-limiting for tumor formation. Clearly, since most WTs show conversion to a bipaternal epigenotype at *IGF2/H19,* either via LOH or via the non-LOH pathway, and since most are not bilateral or multifocal, this type of lesion is unlikely to be rate-limiting. In our series, only 4 of the 40 tumors with biallelic *H19* DNA hypermethylation were bilateral and the single familial case of WT in our series retained 11p15.5 heterozygosity and was positive for *H19* RNA expression.

If loss of an imprinted tumor suppressor gene is not rate-limiting for tumor formation, then this loss might be expected to be observed both early and late in tumorigenesis. It has recently been shown that a small subset of patients with WT manifest partial "mosaic" 11p15.5 LOH either in their non-neoplastic kidney, in their peripheral blood, or in both tissues, and yet these patients also did not develop multifocal tumors [33]. This type of early loss of chromosome 11p15.5 alleles were seen in the peripheral blood of case pw445 in our series, a patient with unilateral WT and no signs of BWS (Fig. 3). The non-LOH counterpart of this situation is the early epigenetic inactivation of *H19* in our cases 428 (WT3) and 511 (WT21) [10] (Fig. 1). Despite widespread *H19* inactivation and, in at least one of the patients, *IGF2* mRNA activation, in the non-neoplastic kidney parenchyma [10], neither patient had bilateral or multifocal tumors. The converse situation of 11p15.5 LOH late in tumorigenesis was seen in case 513 [WT15 in 10]. This tumor expressed substantial amounts of H19 RNA [10] but on both Southern blot and PCR analysis this tumor showed a slight but reproducible loss of the signal intensity of the active *H19* allele (Fig. 4A,B). Thus, a minor clone of cells with 11p15.5 LOH had developed late in the progression of this tumor.

DISCUSSION

What process might account for the epigenetic inactivation of *H19* in WTs? At least three possibilities come

TABLE I. Chromosome 11p15.5 allelic status, *H19* DNA methylation and *H19* RNA expression in WTs†



†Cases which were bilateral or familial or which had unusual histological features (anaplastic WT, WT with component of embryonal adenoma) are indicated. The tumors designated pw844 and pw1303 were from opposite kidneys of a bilateral case. *H19* RNA is scored as: − if the level was more than 20-fold reduced by densitometry from that observed in 18 to 20-week fetal kidney; (+) if more than 10-fold reduced; and + if equivalent or greater than fetal kidney levels. Some of the loci were evaluated with several markers (*IGF2* ApaI polymorphism and CA-repeat; *H19* CfoI, AatII, RsaI, AluI, AviII and TaqI polymorphisms; *HRAS* TaqI polymorphism and CGG-repeat) and were scored as heterozygous if any one of these analyses showed two allelic bands. *Partial (>50%) LOH in tumor. **Scored as retention of heterozygosity but slight (<25%) loss of one allelic band in analysis of tumor DNA. #Partial LOH in peripheral blood. The genetic distances between D11S988 and HRAS are from the Version 3.0 Sex-Averaged Framework Map extended with Version 3.0 CHLC markers (Genome Database). nd, not determined.

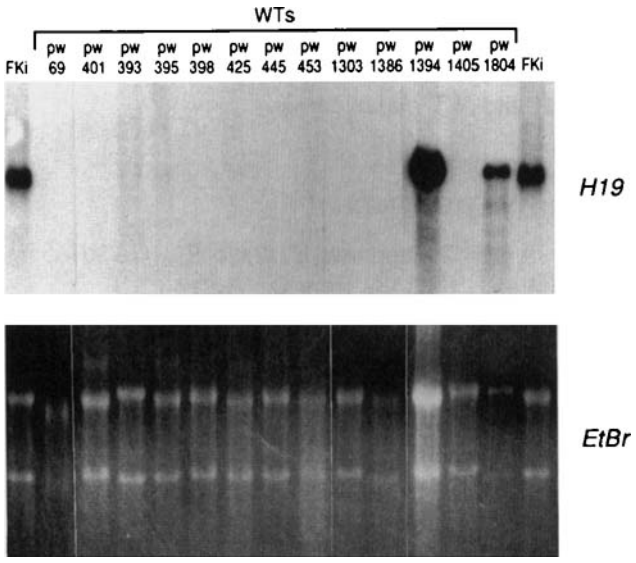


Fig. 2. Northern blot analysis of *H19* RNA in WTs and fetal kidney (FKi). EtBr, ethidium bromide staining of 28S and 18S ribosomal RNA. The lane for case pw1394 is overloaded.

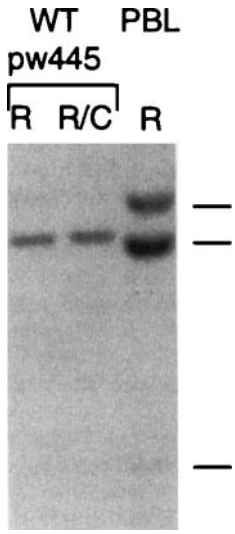


Fig. 3. Chromosome 11p15.5 LOH in peripheral blood. DNAs were digested with RsaI (R) or RsaI + CfoI (R/C). The blot was hybridized with the *H19* exon 3–5 probe. The tumor shows complete LOH with loss of the upper RsaI allelic band. The remaining allele is fully resistant to digestion with CfoI. The peripheral blood (PBL) shows partial loss of this same allele.

to mind. The most trivial explanation would invoke the action of an altered transcription factor in the tumor cells on the *H19* and/or *IGF2* promoters. For example, the chromosome 11p13 *WT1* gene product, a zinc-finger transcription factor which is mutated or deleted in at least 5–10% of WTs, has been shown to have the ability to transcriptionally repress the *IGF2* promoter in cotransfection assays [34]. If a similar transcription factor gene were to reside at chromosome 11p15.5, its mutation or functional deletion might account for the findings, but as yet there is no positive evidence for this possibility. Also, the postulated transcription factor would have to normally activate *H19* and repress *IGF2,* but the *IGF2* and *H19* promoters normally appear to respond in the same direction to the same set of transcriptional activators, as indicated by the remarkably concordant patterns of expression of these two genes [23]. A second possibility is that there might be an actual physical transfer of the DNA methylation imprint from the inactive paternal copy of *H19* to the active maternal copy, i.e., allelic trans-sensing, in the tumor precursor cell. In principal this could occur in the context of a DNA recombination intermediate such as a double Holliday junction structure, with the cytosine DNA methyltransferase acting on transient DNA heteroduplexes. The biallelic activation of *IGF2* would then follow as a consequence of the inactivation of *H19* via the enhancer competition mechanism.

A third possibility is that the observed epigenetic changes at the *H19* and *IGF2* genes might reflect a broader disruption of chromosome 11p15.5 functional imprinting, based on some somatic event in the tumor precursor cell which "resets" the imprint. It has recently been proposed that the subset of WTs with 11p15.5 LOH and those without LOH may both derive from daughter cells which have undergone a fundamentally similar genetic recombination event, i.e., mitotic crossing over [35]. Depending on the timing of the DNA crossover and the pattern of subsequent strand segregation, such an event always leads to transfer of markers between chromosomes but need not necessarily lead to loss of heterozygosity. By placing distal 11p15.5 maternal alleles in physical contiguity with a proximal 11p15.5 paternal "imprinting center" it might, however, consistently lead to disruption of imprinting.

What can be concluded regarding the status of *IGF2* and *H19* as candidate dominant oncogene and tumor suppressor gene, respectively, in WTs? WTs tend to express *IGF2* mRNA at levels equivalent to or exceeding the already high levels found in the fetal kidney, and inactivation of *H19* is clearly associated with biallelic activation of *IGF2* transcription. The biggest stumbling block in the "*IGF2* hypothesis" is the lack of a demonstration that Igf-2 protein expression is in fact high in WTs and that the protein levels correlate with mRNA levels. Two preliminary reports suggest that this is not the case [36,37] and imply that much of the *IGF2* mRNA in WTs may be "sterile," but more studies are needed. Timing might be important here; perhaps a WT precursor cell expresses high Igf-2 protein but the fully developed tumors do not. With regard to *H19,* aside from the obvious problem that we do not know biochemically how an untranslated RNA might affect cell growth, the fact that a significant percentage of adult carcinomas can express high levels of *H19* RNA [38,39] and the recent report that mice lacking *H19* RNA do not develop tumors [40] have not supported the contention

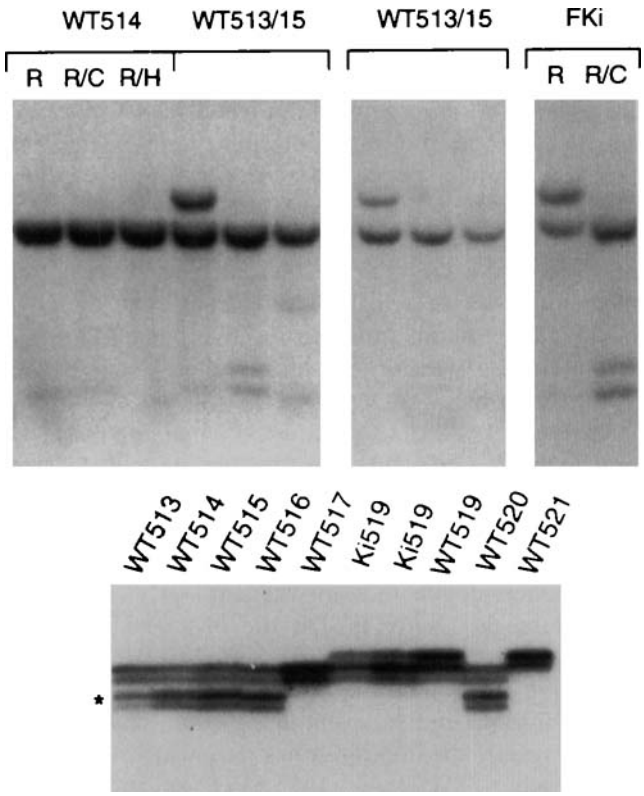


Fig. 4. Incipient chromosome 11p15.5 LOH late in tumor progression. **Above:** Southern blot showing partial loss of the upper RsaI *H19* allele in case 513 [WT15 in ref. 10]. The middle panel shows a lighter exposure. The upper allele is the active one in this case, as shown by its complete digestion with the methylation-sensitive enzymes. The tumor from case 514 retained 11p15.5 heterozygosity but the patient was homozygous for the *H19* RsaI marker. There is biallelic *H19* DNA hypermethylation in this case, with complete resistance to digestion with methylation-sensitive enzymes. R, RsaI; R/C, RsaI + CfoI; R/H, RsaI + HpaII; FKi = Fetal Kidney. **Below:** Analysis of the HRAS CGG-repeat polymorphism. In this analysis each allele is seen as a doublet and in cases which retain heterozygosity the lower alleles are reproducibly slightly more intense than the upper alleles. There is subtle partial loss of the lower allele in case 513 (asterisk). There is more complete LOH in case 519 (compare Ki519 with WT519).

that *H19* is a tumor suppressor gene. But these observations do not effectively argue against this possibility either, since there are numerous examples of tumors which express high levels of tumor suppressor gene products (high levels of apparently normal *WT1* gene product in mesotheliomas are one example [41]) and mice have never been observed to develop embryonal kidney tumors, even in the setting of hemizygous germline *Wt1* deletions [42].

For both *IGF2* and *H19,* as well as for *KIP2,* proof of a critical role in Wilms' tumorigenesis may depend on finding localizing DNA lesions in these genes in WTs. Additional mapping of 11p15.5 genomic imprinting [28] will also be essential to determine whether these are the only genes in this region whose functional imprinting is altered in WTs. While these important issues remain to be settled, it is clear that disruption of the expression of imprinted genes is the most consistent molecular abnormality identified in WTs to date and it is likely that further study of this phenomenon will continue to yield important insights into WT pathogenesis.

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COMMENTARY

Moulton et al. discuss genomic imprinting, an important phenomenon in tumorigenesis in general and in the development of Wilms' tumor in particular. Genomic imprinting (or imprinting) encompasses a process in which gene expression is observed to be specific to parent-of-origin. In other words, imprinted genes are preferentially expressed from one parental allele, with genes silenced on the paternal allele referred to as "paternally imprinted" and those silenced on the maternal allele as "maternally imprinted" genes. This is important since it explains why loss of a specific (for instance maternal) allele has been observed in certain tumors: only loss of the active allele will result in a phenotype; for example, loss of the active tumor suppressor allele results in uncontrolled growth, while loss of the imprinted (inactive) allele has no visible effect. This manuscript describes that in most Wilms' tumors, two genes on chromosome 11p15 (*H19* and *IGF2*) are reciprocally imprinted and that in most cases the candidate tumor suppressor gene *H19* is inactive, while the candidate dominant oncogene *IGF2* is actively expressed both from the maternal and paternal allele. The mechanisms leading to this situation are described.